Culture Environment Regulates Amino Acid Turnover and Glucose Utilisation in Human ES Cells

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Abstract

Human embryonic stem (ES) cells have been proposed as a renewable source of pluripotent cells that can be differentiated into various cell types for use in research, drug discovery and in the emerging area of regenerative medicine. Exploitation of this potential will require the development of ES cell culture conditions that promote pluripotency and a normal cell metabolism, and quality control parameters that measure these outcomes. There is, however, relatively little known about the metabolism of pluripotent cells or the impact of culture environment and differentiation on their metabolic pathways. We have examined the effect of two commonly used medium supplements and cell differentiation on metabolic indicators in human ES cells. Medium modifications and differentiation were compared in a chemically defined and feeder independent culture system. Adding serum increased glucose utilisation and altered amino acid turnover by the cells, as well as inducing a small proportion of the cells to differentiate. Cell differentiation could be mitigated by inhibiting p38 MAPK activity. The addition of Knock Out Serum Replacer® also increased glucose uptake and changed amino acid turnover by the cells. These changes were distinct from those induced by serum and occurred in the absence of detectable differentiation. Induction of differentiation by BMP4, in contrast, did not alter metabolite turnover. Deviations from metabolite turnover by ES cells in fully defined medium demonstrated that culture environment can alter metabolite use. The challenge remains to understand the impact of metabolic changes on long-term cell maintenance and the functionality of derived cell populations.
Introduction

Pluripotent cell lines derived from the human blastocyst, human embryonic stem (ES) cells, were first described in 1998 (Thomson et al. 1998; Reubinoff et al. 2000). In the intervening years the isolation of more than 200 individual human ES cell lines has been reported (reviewed in (Adewumi et al. 2007)). Despite the varied genetic background of these isolates, and the diversity of clinics and laboratory conditions involved in their isolation, these cells show a remarkable consistency with regards to pluripotent cell marker expression (Adewumi et al. 2007). They retain a broad differentiation potential in culture, capable of generating cell populations consistent with derivatives of the three primary germ lineages and the extraembryonic endoderm. The cells can be propagated indefinitely while maintaining a normal karyotype and express genetic markers and cell surface antigens correlated with pluripotency, including OCT4, NANOG, TDGF, GDF, DNMT3B, FGF4, GCTM2 and TRA1-60/TRA1-81 (Adewumi et al. 2007). To date, the evaluation of human ES cell lines has focused on assessing parameters that are associated with cell function, or pluripotency. In contrast, little emphasis has been placed on characterizing the physiology of these cells. Given that appropriately regulated metabolism is fundamental to cell function and viability, it is paramount that a greater understanding of metabolic events that occur during stem cell derivation, culture and differentiation is developed. Application of this knowledge will underpin the development of propagation media and protocols that ensure maintenance of cell state and cell physiology. Cell physiology markers are not currently employed for the appraisal of human ES cells in culture or in the development of human ES cell culture medium.
The physiology of the early embryo, from which ES cells are routinely derived, has been extensively characterized. Perturbation of the metabolism of the preimplantation embryo significantly impairs subsequent embryo implantation and development (Lane and Gardner 1996; Gardner 1998). Embryonic metabolism is sensitive to the composition of culture medium. Serum, a common addition to culture media, has been shown to damage blastomere ultrastructure, specifically affecting mitochondrial state and function (Thompson et al. 1995). As a consequence, oxidative function is compromised, gene expression and imprinting affected and embryo development impaired (Khosla et al. 2001). Subsequent fetal development in laboratory and domestic animals is associated with compromised outcomes, demonstrating that altered physiology at the preimplantation embryo stage, induced by the culture medium, has significant downstream effects (Thompson et al. 1995; Gardner 1998; Lane and Gardner 2005).

Early alterations in physiology likely impact later developmental through interaction with the epigenetic programming that occurs at this stage in development and causing the establishment of epigenetic marks that impinge on later life (Donohoe and Bultman 2012). The development of culture media designed to maintain normal embryo physiology and function, coupled with the development of metabolic markers of embryonic viability (Gardner 1998; Sakkas and Gardner 2005; Gardner 2011) has resulted in improvements in embryo quality in culture and concomitant improvements in outcomes from assisted reproductive technologies in laboratory and domestic animals and in humans (Oddens 2006).

Understanding the environmental requirements for the optimal maintenance of human ES cells in culture will require knowledge not only of the signaling pathways that regulate
pluripotency and differentiation but also, and of equal significance, of the specific
nutrient requirements of the cell and how the metabolome is affected by different
environmental conditions and states of differentiation. One of the limitations of
characterizing human ES cell physiology is the diversity of medium and supplements that
are routinely used in the culture of these cells coupled with a lack of systematic
assessment of how human ES cells respond to the culture environment. Here we
characterize glucose use and amino acid turnover in human ES cells in culture; both of
these parameters have been used to assess embryo quality and can be correlated with
improved embryo outcome (Lane and Gardner 1996; Houghton et al. 
2002; Brison et al. 
2004; Gardner 2011; Gardner et al. 2011). The addition of serum (20%), or Knockout
Serum Replacer (Knockout™ SR; Life Technologies; 20%), to a chemically-defined
human ES cell culture system resulted in altered amino acid turnover, increased uptake
and metabolism of glucose and increased cell loss from the population. These changes
did not correlate with the level of pluripotency, extent of differentiation in the cultures or
with changes in cell proliferation. In contrast, induction of differentiation by BMP4 in
this system had little effect on glucose uptake and metabolism or amino acid turnover.
These data demonstrate that in human ES cells metabolic indicators can be affected
significantly by the culture environment and suggest that the cells can experience stress in
response to sub-optimal culture conditions. Before the full realization of the clinical and
commercial potential of human ES cells can be achieved the impact of altering cell
metabolism on viability and function needs to be understood.
Materials and Methods

Human ES cell culture

The human ES cell line, MEL-2 (Australian Stem Cell Centre), was cultured in mTeSR1 medium (STEMCELL Technologies) (Ludwig et al. 2006a; Ludwig et al. 2006b), on human ES cell-qualified Matrigel™ (BD Biosciences), as per the manufacturer’s instructions. Cells were passaged every 7 days using Dispase (STEMCELL Technologies) at 37°C. Cell clumps were resuspended in mTeSR1 medium and plated at a ratio of 1:6 to 1:10 in 6 or 12 well cluster dishes (Falcon, Becton, Dickinson and Company). Cells were cultured at 37°C in 5% CO₂ in ambient air in a Galaxy R incubator (RS Biotech). Foetal calf sera (sourced from Invitrogen and Hyclone) and Knock-out Serum Replacer (Knockout™ SR; Invitrogen) were added after 72 hours (FCS and Knockout™ SR) or 144 hours (FCS) and replaced daily until day 7, or 168 hours. SB203580 (Sigma Aldrich) was added after 72 hours to a concentration of 10 µM; a commensurate volume of diluent (DMSO) was added to controls. Medium was replaced at a similar time each day. Each experiment was set up in triplicate and triplicate wells were treated as a covariate in statistical analysis. On completion of cell culture and after medium collection cells were incubated with TrypLE Select (Life Technologies) for 8 minutes at 37°C, triturated to a single cell suspension and counted on a haemocytometer. Images of human ES cells were captured on an Olympus IX50 inverted microscope.

Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from human ES cells with an RNAqueous-4PCR Kit (Ambion) according to the manufacturer’s instructions. RNA was DNaseI treated (Ambion) and cDNA was synthesized with M-MLV Reverse Transcriptase (Promega) and oligo(dT)
primers (Promega). Real-time PCR reactions were set up in triplicate using ABsolute
Blue QPCR SYBR Green Mix (Thermo Fisher Scientific) and run on an MJ research
thermocycler with a Chromo4 Continuous Fluorescence Detection System (MJ Research)
in the following sequence: 95°C for 15 minutes, followed by 40 cycles of denaturation at
95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30
seconds. The sequences of primers are listed in Table S1. The relative concentration of
each gene was normalized to $\beta$-ACTIN and analysed using Q-Gene software package
(Simon 2003).

**Analysis of metabolic activity**

Media, collected from human ES cells 96 or 24 hours after addition of FCS or
Knockout™ SR and 24 hours after the culture medium was renewed, were snap-frozen in
liquid nitrogen and stored at -80°C. Media only controls comprised media that had been
incubated for 24 hours at 37°C in a well pre-coated with Matrigel™. Nutrient
consumption and metabolite production rates, expressed as fmol/cell/hour, were
calculated using the following formula: Consumption or production rate = $C_0 - C_1$ / # of
cells X # of hours where $C_0$ is the nutrient or metabolite concentration in control media
and $C_1$ is the nutrient or metabolite concentration in the experimental media. Cell refers
to the final cell number in each well. Final cell number in mTeSR1 + serum and mTeSR1
+ KOSR was adjusted to reflect the cell loss seen in these conditions, estimated to be a
total of 11% over a 24 hour period.

**Measurement of carbohydrate consumption and production**

Glucose concentration in the media was estimated using an enzymatic assay linked to
NADPH production. One μL of medium, previously diluted 1:10 in water, was added to
10 μL of glucose reagent (3.7 mM MgSO₄·7H₂O, 0.6 mM NADP⁺, 0.5 mM ATP, 0.5 mM dithiothreitol, 12 U hexokinase/mL, and 6 U G6PDH/mL in EPPS buffer with pH 8.0) (Gardner and Leese 1990). Similarly, lactate concentration in the media was estimated using an enzymatic assay linked to NADH production. Two μL of medium, previously diluted 1:10 with water, were added to 10 μL of lactate reagent (4.76 mM NAD⁺, 100 U LDH/mL, and 2.6 mM EDTA in glycine-hydrazine buffer with pH 9.4) (Gardner and Leese 1990). Fluorescence was measured using a Nanodrop 3300 Fluorospectrometer (Thermo Fisher Scientific).

The percentage of glycolysis was calculated based on one mole of glucose yields two moles of lactate (% of glycolysis = # of moles of lactate / (# of moles of glucose x 2)).

Measurement of amino consumption and production with Liquid Chromatography-Mass Spectrometry (LC-MS)

The concentration of amino acids in the culture media was measured by LC-MS. All materials used for derivatisation of amino acids in the media and amino acid standards were from Sigma-Aldrich. To derivatise amino acids, 10 μL of medium, diluted 1:30 in water, was added to 70 μL of borate buffer (200 mM, pH = 8.8), followed by the addition of 20 μL of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) solution (3 mg of AQC dissolved in 1 mL of dry acetonitrile). Reactions were shaken at 55°C for 10 minutes before LC-MS analysis on an Agilent 6410 LC-ESI-QQQ equipped with an Agilent 1200 Series LC system (Agilent Technologies). A Multiple Reaction Monitor was developed for each individual amino acid. Conditions were capillary temperature 300°C, capillary voltage 4000V, and gas flow rate 10 L/minute. The concentration of each amino acid was obtained in every sample.
159  *Determination of amino acid turnover:*

160  The measurement of turnover was adapted from the approach of Houghton et al. (Houghton *et al.* 2002), and represents the sum, in fmol/cell/hour of amino acids consumed or released into the culture medium. Total amino acid turnover was the sum of amino acid change in fmol/cell/hour.

164  *Immunofluorescence*

165  Human ES cells were cultured on Matrigel™-treated glass coverslips in mTeSR1 and supplements as described in the text. Cells were washed with PBS and fixed with 4% PFA. Fixed monolayers were made permeable with PBS/0.25% TritonX, blocked with 1% BSA or donkey serum. Antigens were detected with antibodies directed against NANOG (R&D Systems) or phospho-histone H3 (Ser10) (Cell Signaling Technologies) in combination with an Alexa-Fluor 488 conjugated anti-goat antibody (NANOG; Life Technologies) or an Alexa-Fluor 568 conjugated anti-rabbit antibody (Phospho-Histone H3 (Ser10); Life Technologies); DNA was detected with DAPI. Images were taken on an Olympus BX50 microscope with an Olympus F-viewII digital camera (NANOG) or a confocal microscope (Phospho-Histone H3 (Ser10). Percentage of mitotic cells was calculated as the percentage of DAPI nuclei positive for phospho-histone H3 (Ser10) in randomly captured fields.

177  *Statistical analysis*

178  Gene expression and carbohydrate use were analysed by Student’s two-tailed t-test. Analysis of the variance in amino acid use in control samples was performed using ANOVA. Analysis of amino acid use in experimental samples, in comparison with the controls, was performed using the R statistical software package with in-house
customized scripts. A logarithm transformation was applied to all metabolite concentration measurements to minimize heteroscedastic noise to ensure a Gaussian data distribution prior to analysis. A two-tailed paired Student’s t-test was used to calculate significant differences between the means of metabolite concentration responses. Manhattan Hierarchical Cluster Analysis was undertaken to confirm reproducibility of sample replicates and to determine the relationships between metabolites based on a measure of distance similarity. Pearson Correlation Analysis was conducted on the logarithm transformed metabolite measurements to confirm relationships of metabolite consumption. For all analyses, a $P$-value of less than 0.05 was considered statistically significant.
Results

Serum induces sporadic differentiation of human ES cells.

The human ES cell line MEL2 (Australian Stem Cell Centre) was cultured in mTeSR1 for 72 hours followed by 96 hours in mTeSR1 supplemented with one of two foetal calf sera at a concentration of 20%. The sera used in this experiment had been selected for use with mouse ES cells and shown to elicit low levels of cell differentiation (Rathjen and Rathjen 2003) (Dr Jeff Mann, Murdoch Childrens Research Institute, Parkville, Victoria; unpublished). In mTeSR1, human ES cells grew in tightly-packed colonies with smooth edges (Figure 1A)(Ludwig et al. 2006b). The addition of serum resulted in a loosening of the cells at the edge of the colonies and formation of a border region comprising fibroblast-like cells (Figure 1B). Comparable alterations in morphology were observed with both sera tested (Figure 1B, C).

Expression levels of the pluripotent markers OCT4 and NANOG in cells cultured in serum remained high and similar to cells cultured in mTeSR1 alone, although a small but consistent reduction in NANOG expression was observed with both sources of sera (Figure 1D). This reflected the substantial pool of pluripotent cells in cultures, as shown by immunocytochemistry (Figure 1E). Only cells at the edge of the colonies had reduced NANOG protein levels, while the majority of the cells within the colonies remained NANOG positive. Markers of differentiation, BRACHYURY and GATA4, were elevated in cells cultured in serum. The fold increase of these markers was variable between experimental repeats suggesting that the proportion of differentiated cells in populations cultured in serum was unpredictable. The reduction in NANOG expression, expression of differentiation markers and morphology changes, however, suggested a heterogeneous
population of cells in serum in which the frequency of differentiated cells was increased.

A similar heterogeneity has been seen in mouse ES cells cultured in serum-supplemented medium when compared to those cultured in defined medium (Marks et al. 2012).

Differentiation of mouse ES cells in response to serum requires p38 MAPK activity (CY and JR unpublished). P38 MAPK activity can be inhibited pharmacologically with SB203580 (4-(4´-fluorophenyl)-2-(4´-methylsulfinylphenyl)-5-(4´-pyridyl)-imidazole), which inhibits p38α, p38β and p38β2 homologues by competing for ATP binding pockets (Cuenda et al. 1995). The expression of pluripotent and differentiated markers were analysed in cells cultured in mTeSR1 + FCS and compared to expression in cells cultured in mTeSR1 + FCS + SB203580. Expression of OCT4 was significantly increased, and BRACHYURY and GATA4 significantly decreased, in cells cultured in mTeSR1 + serum + SB203580 (Figure 1F). These data confirm an increase in differentiation in cells cultured in serum and suggest a requirement for p38 MAPK in the process.

Serum induces alterations in the metabolic activity of human ES cells in culture.

Carbohydrate use by human ES cells cultured in mTeSR1 was compared to that of cells exposed to serum for 24 and 96 hours. The measured concentration of glucose and lactate in mTeSR1 and mTeSR1 + serum can be found in Table S2. Cells cultured in serum consumed more glucose and produced more lactate than controls (Table 1). Glucose consumption approximately doubled with 96 hours of exposure to serum. Although more glucose was being metabolised by cell cultures exposed to serum, the percentage of glucose metabolised glycolytically in these cells was not altered when compared to cells cultured in mTeSR1 (Table 1).
In the published formulation of mTeSR the medium includes amino acids in a range of concentrations standard for tissue culture maintenance. Repeated measurement of mTeSR confirmed the presence and the concentration of amino acids in the medium, and provided confidence that the technology used could reliably measure amino acids within medium samples (Figure S1). In mTeSR1 + serum the concentrations of alanine, glutamic acid and glycine were significantly increased when compared to mTeSR (Table S3). The concentration of amino acids in medium used for culturing human ES cells was determined and compared to unused medium. Cells cultured in mTeSR1 or mTeSR1 + serum produced alanine, glutamic acid, proline and ornithine (Figure 2A). The production of glutamic acid was significantly higher from cells cultured in serum (Figure 2A). Human ES cells consumed arginine, cysteine, glutamine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine and γ-aminobutyric acid (GABA) (Figure 2A). The addition of serum to the culture medium significantly increased the consumption of the majority of these amino acids (Figure 2A). Overall, the addition of serum increased the total turnover of amino acids by the cells 1.9-fold to 106 fmol/cell/hour (Figure 2B), comprised of a 1.7-fold increase in amino acid production and a 1.9-fold increase in amino acid consumption.

**Differentiation of human ES cells in response to BMP4 does not alter the carbohydrate usage or amino acid metabolism.**

Metabolism in pluripotent cells and somatic cells differs, suggesting that the changes in glucose use and amino acid turnover in serum could arise from differentiated cells in the population. Alternatively, changes in metabolomic activity could result from serum-induced changes in the activity of all cells. The differentiation of human ES cells was
induced by the addition of BMP4 to mTeSR1 for 4 days (Hughes et al. 2009) and carbohydrate usage and amino acid metabolism were measured. Like serum, BMP4 induces expression of Brachyury and Gata4 (Kobayashi et al. 2008; Hughes et al. 2009) (CY and JR unpublished) and is likely to result in a repertoire of cells that overlaps those formed in serum.

As expected, the addition of BMP4 resulted in an overtly differentiated colony morphology with each colony consisting of a small core of pluripotent, NANOG positive, cells surrounded by cells of an overtly differentiated phenotype (Figure 3A,B and data not shown). Previous analysis has demonstrated the decreased expression of NANOG and increased expression of differentiation markers in human ES cells exposed to BMP4 (Hughes et al. 2009). The analysis of spent medium from these cultures did not show any changes in the consumption of glucose, production of lactate or percentage glycolysis when compared to cells cultured in mTeSR1 (Table 1). Similarly, the addition of BMP4 had little effect on production or consumption of the amino acids tested, with differences detected only in consumption of tryptophan and production of glutamic acid and proline, and no overall change in amino acid turnover (Figure 3C, D).

Knockout™ SR induces metabolic changes in human ES cells.

Knock-out serum replacer (Knockout™ SR; Life Technologies) is commonly used as a medium supplement for the growth and differentiation of human ES cells (Adewumi et al. 2007). Knockout™ SR was added to mTeSR1 and the effects on human ES cell growth and metabolism were measured. Addition of 20% Knockout™ SR to mTeSR1 resulted in minor alterations in colony morphology, with some fibroblast-like cells
appearing at the edge of the colonies (Figure 4A, B). The change in colony morphology was, however, much reduced in comparison to cells exposed to serum or BMP4. Similarly, gene expression analysis of these cells did not detect any reduction in \textit{NANOG} expression or increase in the expression of the differentiation markers (Figure 4C).

Carbohydrate use by cells exposed to Knockout™ SR for 96 hours was compared to use by cells cultured in mTeSR1 (Table 1). Knockout™ SR increased the consumption of glucose and production of the lactate by approximately 1.4 fold. These changes were not accompanied by a change in the percentage of glucose metabolised glycolytically.

The measurement of amino acid concentrations in medium containing Knockout™ SR showed several amino acids, isoleucine, phenylalanine, proline, serine, threonine, tryptophan and valine, to be present at unusually high concentrations when compared to mTeSR1 (Table S4). These concentrations were not, however, unexpected when the preferred embodiment of the medium contained within the patent describing the formulation of Knockout™ SR is considered (Price 1998). The profile of amino acids produced and consumed from cells exposed to Knockout™ SR was significantly different from that of cells cultured in mTeSR1, and from cells exposed to serum or BMP4. Cells in Knockout™ SR produced glycine, isoleucine, phenylalanine, threonine, valine and GABA (Figure 5A). In contrast, cells cultured in mTeSR, mTeSR + serum and mTeSR + BMP4 consumed these amino acids. The production of alanine, glutamic acid, and proline, and the consumption of lysine and serine, was significantly increased by the addition of Knockout™ SR (Figure 5A). Overall turnover of amino acids by cells cultured in Knockout™ SR was increased 1.9-fold, to 109 fmol/cell/hour (Figure 5B).

Although this value was similar to the increase in amino acid turnover seen from cells
cultured in serum, the changes in production and consumption contributing to this were
different, with a 4.6 fold increase in amino acid production and 12% reduction in amino
acids consumed.

Cell proliferation is not affected by the addition of serum or Knockout™ SR.

The addition of serum or Knockout™ SR potentially alters the growth characteristics of
human ES cells and affects the requirement of these cells for nutrients. The proportion of
cells in mitosis, identified by staining for phospho-histone H3 (Ser10), in human ES cells
cultured in mTeSR1 or mTeSR1 supplemented with serum or Knockout™ SR was
measured. No significant difference in cell proliferation was detected (Table 2). The
addition of serum or Knockout™ SR, however, did reduce the number of cells in culture
by approximately 50% at the time of assay (Table 3).
Discussion

The establishment of human ES cells by Thomson and colleagues in 1998 has resulted in a dramatic increase in publications analysing the genetics, epigenetics and differentiation of these cell lines, including multiparticipant studies that compared these characteristics across multiple human ES cell lines (Adewumi et al. 2007; Akopian et al. 2010; Amps et al. 2011). In contrast, there has been relatively little investigation of the physiology of these cells, even when the suitability of medium formulations for human ES cell culture is being tested (Akopian et al. 2010). Cellular metabolism is fundamental to embryo viability and cell function (Gardner 1998; Lane and Gardner 2005), and has been shown to be adapted in cells as they acquire characteristics of cancer and disease (Fritz and Fajas 2010; Chang and Wei 2011). If the clinical and commercial potential of human ES cells is to be realized, a greater understanding of metabolic activity and regulation of metabolic processes during stem cell derivation and differentiation will need to be acquired and media formulations that support an appropriate metabolic state developed. Physiological markers of pluripotency will find future applications in the validation and accreditation of existing and newly isolated human ES cell lines.

In this study mTeSR1 was chosen as a standard human ES cell culture system against which to compare the effect of supplements and differentiation on metabolism. mTeSR1 supports the growth of human ES cells in the absence of a feeder layer that would otherwise complicate analysis (Ludwig et al. 2006a; Akopian et al. 2010). mTeSR1 has been disclosed, which allows experimental measurements to be compared with the published formulation (Ludwig et al. 2006a). The medium is based on a widely used medium, DMEM:F12 mix, and provides salts, nutrients and amino acids at concentrations...
previously determined to be sufficient for the culture of mammalian cells in culture and
extensively used for the culture of mouse pluripotent cells and human ES cells. The
medium contains a high concentration (13.7 mM) of glucose as an energy source. After
24 hours of culture in wells containing near confluent cell colonies the medium contained
approximately one third of starting glucose, a final concentration of between 4 and 5 mM.
The osmolality of supplemented medium remained within the normal range (data not
shown). Medium supplementation will, however, have modified the composition of the
medium and altered the concentration of growth factors in the formulation. Adding 20%
F12 to mTeSR1 did not alter cell proliferation, cell viability, or induce signs of
differentiation (Figure S2; Table S5), suggesting that diluted mTeSR maintained human
ES cells over the time course of this experiment.

The addition of serum or Knockout™ SR did not alter the rate of ES cell division, a
surprising result given the well-established role of serum in stimulating cell proliferation
in culture (Shodell and Rubin 1970). The cell cycle of mouse (Stead et al. 2002) and
human (Kapinas et al. 2013) ES cells is rapid, characterized by truncated gap phases and
distinct in structure from the cell cycle of somatic cells. In mouse, Cdk2, cyclin A and
cyclin E kinases are active throughout the cell cycle and lack the cell cycle periodicity
that is seen in somatic cells (Stead et al. 2002). The inability of serum to stimulate
pluripotent cell proliferation is likely a consequence of the unique characteristics of the
pluripotent cell cycle structure. The addition of the supplements did alter cell viability
and resulted in fewer cells surviving the period of treatment when compared to the
controls.

Glucose uptake and energy use are increased by serum and Knockout™ SR
In mTeSR medium human ES cells used glucose as an energy source, with an estimated 50% of the glucose metabolized to lactate through glycolysis. The high contribution of glycolysis to the energy needs of human ES cells is consistent with previous reports (Prigione et al. 2010; Varum et al. 2011; Zhang et al. 2011). The use of glycolysis when cultured in high oxygen tension (20% O2), known as aerobic glycolysis, is a feature shared with cancer cells and other rapidly proliferating cells in culture, and may reflect the continual proliferation, or self-renewal, of human ES cells (Warburg 1956; Morgan and Faik 1981; Brand et al. 1988; Fox et al. 2005; Lopez-Lazaro 2008; Gardner and Wale 2013; Harvey et al. In press).

The addition of serum or Knockout™ SR increased the amount of glucose used by the cells but did not appear to alter the percentage of the glucose metabolized to lactate, indicating that these supplements increased the energy requirements of the cells. Carbohydrate use by mouse ES cells has been shown to be sensitive to medium composition, with increased glucose uptake from serum-containing medium when compared to medium supplemented with Knockout™ SR (Fernandes et al. 2010b).

Increased glucose uptake in cells cultured in serum could result from a higher flux of glucose in differentiated cells within the population or from increased glucose flux in human ES cells. Increased glucose flux in cells cultured in Knockout™ SR cannot be explained by differentiation, and increasing the proportion of differentiated cells in the human ES cell population, by inducing differentiation with BMP4, did not affect carbohydrate use. These data suggest that the effect of serum and serum replacement on glucose uptake was a consequence of the effect of medium composition. It cannot be excluded that this effect was driven, in part, by dilution of mTeSR1 with the supplements.
Amino acid turnover by human ES cells increases in response to serum and Knockout™ SR

The analysis of amino acid turnover during culture was used as a second indicator of metabolite flux in human ES cells. Cells cultured in serum-supplemented mTeSR1 exhibited a 1.9-fold increased turnover of amino acids when compared to cells cultured in mTeSR1; this increase was a consequence of an approximately 1.9-fold increase in the use and production of amino acids by the population. As with glucose consumption, increased amino acid turnover could be a consequence of the increase in differentiated cells within the population. The addition of BMP4 to mTeSR increased cell differentiation but had little effect on the consumption or production of amino acids, with amino acid turnover similar between cells cultured in mTeSR and BMP4 and mTeSR alone and only minor variations in the turnover of tryptophan, glutamic acid and proline observed.

Cells cultured in mTeSR + Knockout™ SR also showed a 1.9-fold increase in amino acid turnover. Unlike the increased turnover in serum, which affected all amino acids to a similar degree, the increased turnover in cells cultured in Knockout™ SR comprised a prodigious 4.6-fold increase in amino production coupled with a modest 12% decrease in amino acid consumption. These changes occur without any overt differentiation in the population suggesting they arise from a direct effect of Knockout™ SR on the human ES cell. Collectively, these data demonstrate that the interaction of serum and Knockout™ SR with human ES cells resulted in a considerable up regulation of amino acid turnover.
Although similar in scale, increases in turnover resulted from specific cell responses to the medium composition.

**Differentiation and metabolic change are not coincident.**

The metabolism of pluripotent cells and somatic cells are different, which suggests that differentiation will be accompanied by changes in metabolic activity and metabolic pathway use. The differentiation of mouse ES cells has been shown to change carbohydrate use (Kondoh *et al.* 2007; Fernandes *et al.* 2010a), and the differentiation of human ES cells in response to retinoic acid (RA) has been shown to decrease glycolysis (Zhang *et al.* 2011). In contrast, inducing differentiation of human ES cells with BMP4 in mTeSR1 did not change carbohydrate use or glycolytic flux. The timing and regulation of the metabolic changes that accompany differentiation are largely unexplored but changes in metabolism occurred gradually when human ES cells were differentiated with RA (Zhang *et al.* 2011). The unchanged carbohydrate use in BMP4-treated human ES cells after 4 days potentially reflects the gradual nature of change, but may also be impacted by the cell population formed and the medium used. Amino acid turnover was also largely unaffected by human ES cell differentiation. Measuring the intracellular metabolites present in mouse ES cells as they differentiate has shown no change in amino acid concentrations, with the exception of an increase in threonine, and few changes in other metabolites that were measured (Wang *et al.* 2009). These data suggest that in the early stages of pluripotent cell differentiation, as examined here, changes in metabolism lag behind the loss of pluripotence.
Changes in the turnover of individual amino acids can be used as indicators of changes in cell metabolism

Cells cultured in serum supplemented medium consumed significantly more glutamine than those cultured in control medium or mTeSR medium supplemented with BMP4 or Knockout™ SR. Several proliferating cell types, including cancer cells (DeBerardinis et al. 2007), metabolise glutamine to α-ketoglutarate via the formation of glutamate (glutaminolysis), a process that liberates ammonium. The increased glutamine consumption by human ES cells cultured in serum is consistent with increased glutaminolysis and potentially reflects an increased flux of glutamine metabolites through the tricarboxylic acid cycle (TCA) cycle. The increased production of glutamic acid and alanine from cells could be a defensive strategy to alleviate the toxicity of the ammonium formed as a consequence of serum exposure (Morgan and Faik 1981; Weinberg and Chandel 2009). Glutamine consumption was not increased in mTeSR + Knockout™ SR or mTeSR + BMP4 suggesting that neither of these conditions induced increases in glutaminolysis.

The maintenance of pluripotency, and stable glutamine consumption, by Knockout™ SR is consistent with this supplement being more compatible with the physiological needs of human ES cells than serum. The addition of Knockout™ SR to mTeSR, however, significantly altered the concentration of specific amino acids in the medium, increasing their concentration by as much as 30-fold, as is the case for proline (Table S4). The inclusion of Knockout™ SR resulted in profound changes in the way that amino acids were utilized by the cells. In general, amino acids with increased concentrations in Knockout™ SR supplemented medium were produced by cells cultured in Knockout™
SR but consumed by cells cultured in control medium; this was seen for glycine, histidine, isoleucine, phenylalanine, threonine, tryptophan and valine. This was the only medium formulation analysed that elicited changes from the consumption to production of amino acids by the cells. The consumption of serine and production of proline, in contrast, did not change to production and consumption, respectively, despite these amino acids being increased with the addition of Knockout™ SR to mTeSR. For both of these amino acids, however, the magnitude of amino acid turnover was significantly increased. Finally, significant changes were seen in the turnover of alanine, lysine and glutamic acid by cells cultured in Knockout™ SR. The concentrations of these amino acids did not differ between supplemented medium and the control.

The changes in amino acid production detected in cells cultured in Knockout™ SR indicated that multiple biosynthetic pathways were affected, which suggests a model in which a general control process, such as those mediated by mTOR or GCN2 (Bruhat et al. 2000; Kim 2009; Sancak et al. 2010), regulated biosynthetic activation across a number of pathways. Counter intuitively, cells may perceive an amino acid limitation in medium in which amino acid concentrations are not balanced. Elevated concentrations of one amino acid can compromise the ability of a cell surface transporter of amino acids to function and limit uptake of other amino acids. For example, the level of L-proline in Knockout™ SR-supplemented medium is sufficient to inhibit SNAT2 uptake of a number of amino acids, including glycine, alanine, serine, cysteine, glutamic acid, asparagine, histidine and methionine (Tan et al. 2011), and may limit transport of these amino acids into the cell, starving the cell of required nutrients and activating the amino acid sensing pathways. Transporter-mediated amino acid limitation has been shown to regulate mTOR
signaling and activate the GCN2 pathway in vivo (Broer et al. 2011; Pinilla et al. 2011).

Activation of the amino acid sensing pathways, and downstream regulation of biosynthetic pathways, could explain the changes in amino acids production in cells cultured in Knockout™ SR. Although these changes to biosynthesis do not appear to alter the pluripotency of the cells, it is likely changes of this magnitude will impact on other aspects of cell function, including an increase in energy requirements. More analysis will be required before the impact of Knockout™ SR on amino acid biosynthesis in human ES cells can be understood but these data question the suitability of Knockout™ SR in its current formulation for human ES cells culture.

Amino acids can be used by cells for biosynthesis, energy production and as signaling molecules. Recently, a role for proline as a signaling molecule in pluripotent cell differentiation in culture has been described (Washington et al. 2010; Tan et al. 2011).

Human ES cells cultured in mTeSR produced proline and production by cells cultured in mTeSR + serum and mTeSR + Knockout™ SR was significantly increased; in Knockout™ SR proline production was increased approximately 10-fold. Proline production was significantly reduced with BMP4-induced differentiation suggesting that the production of proline is a feature of the pluripotent cells. The elevated concentrations of proline within the medium, either as a consequence of the elevated production of proline by the cells or the addition of proline to the medium, could potentially alter the pluripotent cell state or affect the stability of human ES cells in culture (Pera and Tam 2010; Washington et al. 2010; Tan et al. 2011).

The maintenance of high levels of pluripotency and inhibition of differentiation are the key indicators that have been used to date to drive the development of culture conditions
for human ES cells. Although important these indicators do not reflect the physiology of
the cells; it is clear that the interaction of human ES cells with the medium can induce
significant alterations in carbohydrate use and amino acid turnover without necessarily
impacting on pluripotency. Addition of the supplements also affected cell growth, with
no change in the proliferation rate but significant cell loss from the populations during the
assay potentially reflecting a reduction in cell viability as a result of cell stress. The effect
of altered physiology on human ES cells and their differentiated derivatives is not known
but a wealth of studies of the early embryo suggest that the impact of sub-optimal culture
conditions and altered metabolism can be far reaching and affect long-term development
outcomes, potentially through interaction with the epigenome (Donohoe and Bultman
2012). The work reported here provides baseline measurements of carbohydrate use and
amino acid turnover in human ES cells cultured without feeders and in a fully described,
serum-free medium and establishes a model system that can be used to assess the effects
of environmental modulation on human ES cell metabolism, signaling pathway activity,
transcriptome and epigenome. Further studies are underway to characterise the fluxome
of human ES cells under these conditions, extending the observations reported here to a
more comprehensive range of metabolic pathways and processes.

Like serum, oxygen has been negatively associated with embryo metabolism and
outcome (Wale and Gardner 2012). There is a growing literature describing the effects of
ambient and reduced oxygen tensions on embryonic stem cells in culture. A number of
reports have shown increased pluripotency marker expression (Prasad et al. 2009;
Forristal et al. 2010), improved chromosomal stability (Forsyth et al. 2006), decreased
differentiation (Ezashi et al. 2005; Prasad et al. 2009; Zachar et al. 2010), increased
glycolysis (Kondoh et al. 2007), altered oxygen uptake rates (Abaci et al. 2010),
enhanced derivation of mouse (Gibbons et al. 2006) and human (Peura et al. 2007) ES
cells, and improved generation of iPS cells from mouse embryonic fibroblasts (Yoshida
et al. 2009) in lower oxygen tensions. There are, however, reports that have failed to
demonstrate significant differences between cells cultured in ambient and reduced
oxygen tensions (Ezashi et al. 2005; Forsyth et al. 2006; Prasad et al. 2009; Zachar et al.
2010). Comparison of these studies is hampered by the lack of consistency in other
components of the culture system, which could mask or accentuate the potentially subtle
effects elicited by oxygen, and the variable use of metabolic indicators, some of which
may be insensitive to oxygen tension. To achieve medium optimization we envisage that
the analysis of individual components of the culture environment, like amino acid
concentrations, protein supplements and oxygen tension, will need to be followed by
multifactorial and high-throughput approaches assessing combinations of selected
conditions and a range of sensitive and robust metabolic markers.
535  **Acknowledgements**

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537  assistance with the data analysis, Ms. Natasha Dodge, Ms. Mai Truong and Ms. Jackie
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539  was supported by a Linkage grant from the Australian Research Council and Molecular
540  Biometrics Pty. Ltd. (Connecticut, USA).
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Culture environment regulates human ES cells

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Figure Legends

Figure 1: Serum induces differentiation from human ES cells. A-C. MEL2 human ES cells were cultured in mTeSR1 for 7 days (A) or mTeSR1 for 72 hours followed by mTeSR1 with 20% added FCS for 96 hours (B, C). Serum was from Life Technologies (B) or Hyclone (C). Size bars represent 500µm. D. Human ES cells were collected on day 7 and analysed for the expression of OCT4, NANOG, GATA4 and BRACHYURY by RT-qPCR. n=8 independent repeats. Error bars represent SEM. Data was analysed using a two-tailed Student’s t-test * p< 0.05. E. Human ES cells exposed to Life Technologies FCS for 96 hours were analysed by immunocytochemistry for the presence of NANOG. Nuclei were detected by DAPI. Arrows highlight examples of cells that have lost NANOG expression. F. Human ES cells on day 3 of culture were transferred to mTeSR1+FCS+SB203580 (SB) and cultured for a further 4 days. Cells were collected and analysed for the expression of OCT4, NANOG, SOX2, GATA4 and BRACHYURY by RT-qPCR. Gene expression was normalized to mTeSR1+FCS+DMSO controls. n=3 independent repeats. Error bars represent SEM. Data was analysed using a two-tailed Student’s t-test # p< 0.01.

Figure 2: Exposure to serum changes the use of amino acids in human ES cell cultures. A. Spent medium from the final 24 hours of culture of human ES cells in mTeSR1 for 7 days (□), or mTeSR1 for 72 hours followed by mTeSR1 with 20% Life Technologies FCS for 96 hours (■), was analysed for the presence and concentration of amino acids. Amino acid production (positive values) or consumption (negative values) has been normalised to cell number and is expressed as fmol/cell/hour. n=8 independent
repeats. Error bars represent SEM. Data were analysed using a two-tailed Student’s t-test, *p<0.05; **p<0.01. B. Total amino acid production, consumption and turnover by cells cultured in medium supplemented with serum.

**Figure 3: Differentiation of human ES cells with BMP4 does not result in changes in amino acid usage.** A,B. Human ES cells were cultured in mTeSR1 for 5 days (A) or mTeSR1 for 72 hours followed by 48 hours in mTeSR1 with 30 ng/mL BMP4 (B). Size bars represent 500µm. C. Human ES cells were cultured in mTeSR1 for 7 days or mTeSR1 for 72 hours followed by 96 hours in mTeSR1 with 30 ng/mL BMP4. Spent medium from the final 24 hours of culture was analysed for the presence and concentration of amino acids. Amino acid production (positive values) or consumption (negative values) has been normalised to cell number and is expressed as fmol/cell/hour. n=6 independent repeats. Error bars represent SEM. Data were analysed statistically using a two-tailed Student’s t-test; *p<0.05. D. Total amino acid production, consumption and turnover by cells cultured in medium supplemented with BMP4.

**Figure 4: The addition of 20% Knockout™ SR does not induce detectable differentiation in human ES cells cultures.** A,B. Human ES cells were cultured in mTeSR1 for 7 days (A) or mTeSR1 for 72 hours followed by 96 hours in mTeSR1 with 20% Knockout™ SR (B). Size bars represent 500µm. C. Cells were collected on day 7 and analysed for the expression of OCT4, NANOG, GATA4 and BRACHYURY by RT-qPCR. n=8 independent repeats. Error bars represent SEM. Data was analysed using a two-tailed Student’s t-test; no differences were detected.
Figure 5: Exposure to Knockout™ SR changes amino acid metabolism in human ES cells. A. Spent medium from the final 24 hours of culture of human ES cells in mTeSR1 for 7 days (□), or mTeSR1 for 72 hours followed by mTeSR1 with 20% Knockout™ SR for 96 hours (■), was analysed for the presence and concentration of amino acids. Amino acid production (positive values) or consumption (negative values) has been normalised to cell number and is expressed as fmol/cell/hour. n=12 (6 independent repeats with duplicate wells for each experiment). Error bars represent SEM. Data were analysed using a two-tailed Student’s t-test, *p<0.05; **p<0.01. B. Total amino acid production, consumption and turnover by cells cultured in medium supplemented with Knockout™ SR.
<table>
<thead>
<tr>
<th></th>
<th>Glucose consumption (fmol/cell/hour)</th>
<th>Lactate Production (fmol/cell/hour)</th>
<th>%Glycolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTeSR</td>
<td>160 ± 9</td>
<td>162 ± 12</td>
<td>50.5 ± 2.1</td>
</tr>
<tr>
<td>mTeSR + FCS$^a$; 24 hours$^b$</td>
<td>181 ± 24</td>
<td>187 ± 24</td>
<td>52.3 ± 3.3</td>
</tr>
<tr>
<td>mTeSR + FCS$^a$; 96 hours</td>
<td>357 ± 38**</td>
<td>359 ± 40**</td>
<td>50.5 ± 3.4</td>
</tr>
<tr>
<td>mTeSR</td>
<td>162 ± 12</td>
<td>171 ± 14</td>
<td>53.7 ± 1.4</td>
</tr>
<tr>
<td>mTeSR + BMP4$^c$; 96 hours</td>
<td>163 ± 10</td>
<td>182 ± 14</td>
<td>55.1 ± 3.3</td>
</tr>
<tr>
<td>mTeSR</td>
<td>171 ± 20</td>
<td>179 ± 11</td>
<td>52.1 ± 1.6</td>
</tr>
<tr>
<td>mTeSR + KOSR$^d$; 96 hours</td>
<td>242 ± 16**</td>
<td>251 ± 15**</td>
<td>52.1 ± 2.5</td>
</tr>
</tbody>
</table>

Table 1. Carbohydrate use by human ES cells cultured in mTeSR1 with and without supplementation. $^a$ 20% FCS from Life Technologies. $^b$ All measurements were made on medium taken after the final 24 hours of culture; time in the conditions indicates the length of exposure of the cells to the supplement. $^c$ BMP4 at 30 ng/ml. $^d$ 20% KOSR from Life Technologies.
Table 2: Proliferation of human ES cells, shown as the % of cells positive for phosphorylated histone H3. *20% FCS from Life Technologies.  †20% Knockout Serum Replacer from Life Technologies.  $P$-value determined by Student’s t-test when compared to cells cultured in mTeSR. Analysis was of 20 fields from two independent replicates.

<table>
<thead>
<tr>
<th></th>
<th>% cells in mitosis(^{\pm} \text{s.e.m.} )</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTeSR</td>
<td>3.6 ± .3</td>
<td></td>
</tr>
<tr>
<td>mTeSR + FCS(^a)</td>
<td>4.3 ± .5</td>
<td>.26</td>
</tr>
<tr>
<td>mTeSR + KOSR(^b)</td>
<td>3.0 ± .5</td>
<td>.27</td>
</tr>
</tbody>
</table>

* 20% FCS from Life Technologies.  † 20% Knockout Serum Replacer from Life Technologies.
Table 3: Cell numbers after 4 days of supplement addition. \(^a\) 20% FCS from Life Technologies. \(^b\) 20% Knockout Serum Replacer from Life Technologies. Statistical comparison of raw cell numbers was performed using a paired Student’s t-test, and of cell numbers relative to mTeSR with an unpaired Student’s t-test.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Average cell number</th>
<th>p-value</th>
<th>% mTeSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTeSR</td>
<td>1.92 x 10^6 ± 1.43 x 10^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTeSR + FCS(^a)</td>
<td>9.60 x 10^5 ± 1.83 x 10^5</td>
<td>2.00 x 10^-3</td>
<td>49.8 ± 8.1</td>
</tr>
<tr>
<td>mTeSR</td>
<td>2.09 x 10^6 ± 8.90 x 10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTeSR + KOSR(^b)</td>
<td>1.24 x 10^6 ± 8.40 x 10^4</td>
<td>9.57 x 10^-12</td>
<td>61.0 ± 2.5</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2_Rathjen et al.,
Figure 3_Rathjen et al.
Figure 4

C

Relative gene expression

- OCT4
- NANOG
- GATA4
- BRACHYURY

mTeSR + KOSR

mTeSR

0.2

0.4

0.6

0.8

1.0

1.2

1.4

Figure 4
Figure 5_Rathjen
<table>
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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tr>
<td><strong>B-ACTIN</strong></td>
<td>CGCACCACTGGCATTGTC</td>
<td>TCCTCCTTGATGTCAACGCAC</td>
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<tr>
<td><strong>BRACHYURY</strong></td>
<td>GTGCTGTCCCAGGGCTACAGATG</td>
<td>CCTAAACAGCTCAACTCTACTTTG</td>
</tr>
<tr>
<td><strong>GATA4</strong></td>
<td>CTAAGGCCTGGGTTCATG</td>
<td>TGCTTTAAGTCACCTGTTAG</td>
</tr>
<tr>
<td><strong>NANOG</strong></td>
<td>CAAAGGAAAGAAACACACTTT</td>
<td>TGTGCTGGAGGCTGAGTT</td>
</tr>
<tr>
<td><strong>OCT4</strong></td>
<td>AGCGAACCAGTATCGAGAAC</td>
<td>TTACGAACCACACTGCAC</td>
</tr>
<tr>
<td><strong>SOX2</strong></td>
<td>ATGCACCGCTACAGCTGA</td>
<td>CTTTGCAACCCCTCCATTT</td>
</tr>
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*Table S1: Primer sequences used for qPCR analysis of gene expression.*
<table>
<thead>
<tr>
<th></th>
<th>Expected glucose concentration (mM)</th>
<th>Measured glucose concentration (mM)</th>
<th>Expected lactate concentration (mM)</th>
<th>Measured lactate concentration (mM)</th>
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<tbody>
<tr>
<td>mTeSR</td>
<td>13.7</td>
<td>15.2 ± .4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>mTeSR + FCS</td>
<td></td>
<td>13.4 ± .5</td>
<td></td>
<td>2.4 ± .1</td>
</tr>
<tr>
<td>mTeSR + BMP4</td>
<td>13.7</td>
<td>14.5 ± .3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>mTeSR + KOSR</td>
<td></td>
<td>13.7 ± .4</td>
<td></td>
<td>0</td>
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*Table S2: Measured concentrations of glucose and lactate in control media.*
<table>
<thead>
<tr>
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<th>mTeSR formulation (μM)</th>
<th>mTeSR (μM)</th>
<th>mTeSR + FCS (μM)</th>
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</thead>
<tbody>
<tr>
<td>Ala</td>
<td>137</td>
<td>158 ± 3.54</td>
<td>326 ± 4.6**</td>
</tr>
<tr>
<td>Arg</td>
<td>548</td>
<td>459 ± 8.5</td>
<td>328 ± 5.6</td>
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<tr>
<td>Asn</td>
<td>137</td>
<td>129 ± 2.1</td>
<td>103 ± 1.5</td>
</tr>
<tr>
<td>Asp</td>
<td>137</td>
<td>157 ± 3.4</td>
<td>144 ± 2.8</td>
</tr>
<tr>
<td>Glu</td>
<td>137</td>
<td>157 ± 2.9</td>
<td>301 ± 3.6**</td>
</tr>
<tr>
<td>Gln</td>
<td>2940</td>
<td>2362 ± 38.1</td>
<td>1973 ± 38</td>
</tr>
<tr>
<td>Gly</td>
<td>294</td>
<td>269 ± 7.9</td>
<td>336 ± 10.7**</td>
</tr>
<tr>
<td>His</td>
<td>118</td>
<td>151 ± 1.9</td>
<td>145 ± 2.5</td>
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<tr>
<td>Ile</td>
<td>326</td>
<td>318 ± 6.1</td>
<td>291 ± 3.5</td>
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<tr>
<td>Leu</td>
<td>354</td>
<td>352 ± 7.1</td>
<td>333 ± 3.8</td>
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<tr>
<td>Lys</td>
<td>391</td>
<td>358 ± 5.5</td>
<td>331 ± 3.2</td>
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<tr>
<td>Met</td>
<td>90.6</td>
<td>89 ± 1.9</td>
<td>76 ± 1.2</td>
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<tr>
<td>Phe</td>
<td>169</td>
<td>187 ± 3.5</td>
<td>182 ± 2.1</td>
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<tr>
<td>Pro</td>
<td>216</td>
<td>233 ± 2.8</td>
<td>237 ± 2.1</td>
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<tr>
<td>Ser</td>
<td>294</td>
<td>304 ± 8.9</td>
<td>300 ± 8.1</td>
</tr>
<tr>
<td>Thr</td>
<td>352</td>
<td>343 ± 6.4</td>
<td>301 ± 3.2</td>
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<tr>
<td>Trp</td>
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<td>71 ± 1.3</td>
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<tr>
<td>Tyr</td>
<td>168</td>
<td>174 ± 3.1</td>
<td>160 ± 1.7</td>
</tr>
<tr>
<td>Val</td>
<td>355</td>
<td>355 ± 6.4</td>
<td>355 ± 3.8</td>
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</tbody>
</table>

Table S3: Measured concentrations of amino acids in mTeSR and mTeSR supplemented with 20% Life Technologies FCS, compared to the expected concentrations from the published formulation of the medium. ** p<0.01; significance was tested for those amino acids showing an increased concentration in serum containing medium.
<table>
<thead>
<tr>
<th></th>
<th>mTeSR formulation (µM)</th>
<th>mTeSR (µM)</th>
<th>mTeSR + KOSR formulation (µM)</th>
<th>mTeSR + KOSR (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>137</td>
<td>144 ± 2.5</td>
<td>109.6</td>
<td>123 ± 4.9</td>
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<tr>
<td>Arg</td>
<td>548</td>
<td>581 ± 4.9</td>
<td>438.4</td>
<td>463 ± 16.1</td>
</tr>
<tr>
<td>Asn</td>
<td>137</td>
<td>142 ± 2.2</td>
<td>109.6</td>
<td>113 ± 2.7</td>
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<tr>
<td>Asp</td>
<td>137</td>
<td>139 ± 8.8</td>
<td>109.6</td>
<td>106 ± 4</td>
</tr>
<tr>
<td>Glu</td>
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<td>138 ± 6.2</td>
<td>109.6</td>
<td>105 ± 6</td>
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<tr>
<td>Gln</td>
<td>2940</td>
<td>2614 ±29.5</td>
<td>2352</td>
<td>2065 ± 46.5</td>
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<tr>
<td>Gly</td>
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<td>296 ± 4.5</td>
<td>1176.4</td>
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<td>His</td>
<td>118</td>
<td>116 ± 7.6</td>
<td>1667</td>
<td>925 ± 36.3</td>
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<tr>
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<td>Leu</td>
<td>354</td>
<td>358 ± 6.8</td>
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<td>Lys</td>
<td>391</td>
<td>393 ± 10.7</td>
<td>312.8</td>
<td>308 ± 8.3</td>
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<tr>
<td>Met</td>
<td>90.6</td>
<td>81.6 ± 4.3</td>
<td>465.68</td>
<td>172 ± 4.4</td>
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<td>Phe</td>
<td>169</td>
<td>188 ± 4.1</td>
<td>2847.2</td>
<td>2129 ± 55.6</td>
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<tr>
<td>Pro</td>
<td>216</td>
<td>242 ± 5.9</td>
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<td>6445 ± 144.5</td>
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<td>Ser</td>
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<td>353 ± 9</td>
<td>5038.2</td>
<td>3452 ± 82</td>
</tr>
<tr>
<td>Trp</td>
<td>34.6</td>
<td>47 ± 2.9</td>
<td>812</td>
<td>455 ± 13</td>
</tr>
<tr>
<td>Tyr</td>
<td>168</td>
<td>226 ± 4.9</td>
<td>752</td>
<td>225 ± 4.9</td>
</tr>
<tr>
<td>Val</td>
<td>355</td>
<td>370 ± 7.4</td>
<td>5450</td>
<td>3977 ± 92.6</td>
</tr>
</tbody>
</table>

Table S4: Measured concentrations of amino acids in mTeSR and mTeSR supplemented with 20% Life Technologies KOSR, compared to the expected concentrations from the published formulation of mTeSR \(^{18}\) and mTeSR + KOSR. Expected concentrations for amino acids in KOSR were taken from the preferred embodiment of the supplement \(^{20}\).
Table S5: Proliferation of human ES cells, shown as the % of cells positive for phosphorylated histone H3, in medium conditions used, and final live cell density, estimated be Alamar Blue staining, shown as fluorescent intensity. *P*-value determined by Student’s t-test when compared to cells cultured in mTeSR. Analysis was of 20 fields from two independent replicates (proliferation rate) and 6 replicate wells (final live cell density).

<table>
<thead>
<tr>
<th></th>
<th>% cells in mitosis ± s.e.m.</th>
<th>Fluorescent intensity (relative units) ± s.e.m.</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTeSR</td>
<td>3.6 ± .3</td>
<td>2.95 ± .3</td>
<td></td>
</tr>
<tr>
<td>mTeSR + F12</td>
<td>3.6 ± .3</td>
<td>.86</td>
<td>2.76 ± .2</td>
</tr>
</tbody>
</table>
Figure S2: Comparison of amino acid use in human ES cell cultures across experimental procedures.
A. Spent medium from the final 24 hours of culture of human ES cells in mTeSR1 for 7 days was analysed for the presence and concentration of amino acids. Amino acid production or consumption was normalised to cell number and is expressed as fmol/cell/hour. n=8 (Control 1); n=6 (Control 2); n=12 (Control 3). Error bars represent SEM. Data were analysed using ANOVA, *p<0.05. B. Total amino acid production, consumption and turnover by cells cultured in mTeSR1.

Supplementary figure 1_Rathjen et al.
Supplementary Figure 2: Morphology of human ES cells cultured in mTeSR1 supplemented with 20% Hams F12 medium. Human ES cells were cultured for 3 days in mTeSR1 before being changed into mTeSR1 (A,C) or mTeSR1 supplemented with 20% Hams F12 (B,D) and maintained for a further 4 days. Images of colony morphology were captured in phase contrast at 4 x magnification (A,B) or with fluorescence microscopy after staining for DNA with DAPI (C,D; 20 x magnification). n=3; representative images are shown.